

Original Article

Bis-indole derived nuclear receptor 4A1 (NR4A1) antagonists inhibit TGF β -induced invasion of embryonal rhabdomyosarcoma cells

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Abstract: Transforming growth factor β (TGF β) enhances invasion of breast and lung cancer cells through phosphorylation-dependent nuclear export of the nuclear receptor 4A1 (NR4A1, Nur77). This response is inhibited by the NR4A1 antagonist 1,1-bis(3'-indoly)-1-(p-hydroxyphenyl) methane (CDIM8) and we hypothesized that similar effects would be observed in Rhabdomyosarcoma (RMS) cells. Although some kinase inhibitors block TGF β -induced invasion of embryonal RMS (ERMS) cells, the mechanism differs from breast and lung cancer cells since NR4A1 is extranuclear in ERMS cells. However, CDIM8 blocks basal and TGF β -induced invasion of RD and SMS-CTR ERMS cell lines but not Rh30 alveolar RMS (ARMS) cells. Moreover, this response in ERMS cells was independent of SMAD7 degradation or activation of SMAD2/SMAD3. β -Catenin silencing decreased ERMS cell invasion and CDIM8 induced proteasome-independent downregulation of β -catenin. The novel mechanism of CDIM8-mediated inhibition of basal and TGF β -induced ERMS cell invasion was due to activation of the Bcl-2-NR4A1 complex, mitochondrial disruption, induction of the tumor suppressor-like cytokine interleukin-24 (IL-24) which in turn downregulates β -catenin expression. Thus, the NR4A1 antagonist inhibits TGF β -induced invasion of ERMS cells through initial targeting of cytosolic NR4A1.

Keywords: Invasion, rhabdomyosarcoma, TGF β , β -Catenin, NR4A1

Introduction

Orphan nuclear receptors NR4A1, NR4A2 and NR4A3 are immediate early genes induced by multiple stressors. NR4A receptors play an important role in maintaining cellular homeostasis and in pathophysiology [1]. There is increasing evidence for a functional role for these receptors in metabolic, cardiovascular and neurological functions as well as in inflammation, inflammatory diseases, immune functions and cancer [1-8]. NR4A1 is overexpressed in colon, pancreatic, breast (estrogen receptor positive and negative), and lung tumors. Moreover, high expression of NR4A1 in breast, colon and lung tumors correlates with decreased patient survival [2-7]. The functional activity of NR4A1 in solid tumor-derived cancer cell lines has been extensively investigated by either receptor knockdown or overexpression. The results show that NR4A1 regulates one or more of cancer cell proliferation, survival, cell cycle progres-

sion, migration and invasion in lung, melanoma, breast, lymphoma, pancreatic, colon, cervical, ovarian and gastric cancer cell lines [2-6, 8-16]. Studies in the laboratory have also identified and characterized a series of bis-indole derived compounds (CDIMs) including 1,1-bis(3'-indoly)-1-(p-hydroxyphenyl) methane (DIM-C-pP-hOH, CDIM8) that bind NR4A1 and act as NR4A1 antagonists in solid tumors [2, 5, 6, 11-18]. In solid tumor-derived cancer cell lines, comparable results are observed after NR4A1 silencing or after treatment with the NR4A1 antagonist CDIM8. In contrast to the role of NR4A1 in most solid tumors, expression of this receptor is low in leukemia cells and patients, where there is evidence for tumor suppressor-like activity of this receptor [19, 20].

CDIM8 inhibits growth, survival and migration of RMS cells [15, 16] and these responses are associated with modulation of several genes including survivin, Bcl-2, epidermal growth fac-

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tor receptor (EGFR), other receptor tyrosine kinases and several integrins. In addition, NR4A1 regulates expression of the fusion oncoprotein PAX3-FOXO1 which is a major driver of alveolar RMS (ARMS) and CDIM8 decreased expression of PAX3-FOXO1 and associated downstream genes [16]. Recent studies show that NR4A1 plays an essential role in transforming growth factor β (TGF β) induced invasion of breast and lung cancer cells [3, 17, 18] and CDIM8 inhibits this induced invasion by blocking TGF β -induced nuclear export of NR4A1 [17, 18]. TGF β also induces growth and inhibits differentiation of embryonal RMS (ERMS) cells [21-24]. However, the effects of TGF β on invasion of ERMS or ARMS cells have not previously been investigated. Therefore, in this study, we have investigated the mechanism of action of the NR4A1 antagonist CDIM8 on TGF β -induced invasion of RMS cells. Our results show that TGF β induced the invasion of ERMS cells but not ARMS cells through a novel pathway that involved cytosolic NR4A1 and this invasion was inhibited by CDIM8, which was primarily due to induction of the tumor suppressor-like cytokine interleukin-24 (IL-24).

Materials and methods

Cell lines

RD cells were purchased from the American Type Culture Collection (Manassas, VA). SMS-CTR cells were obtained as a generous gift from Mr. Jonas Nance, Texas Tech University Health Sciences Center-Children's Oncology Group (Lubbock, TX). These cells were grown and maintained at 37°C temperature in presence of 5% CO₂ in either Dulbecco's modified Eagle's medium (DMEM, RD cells), or Iscove's Modified Dulbecco's Medium (IMDM, SMS-CTR cells) in presence of 10% fetal bovine serum (FBS).

Reagents and antibodies

Trypsin, FBS, DMEM, IMDM, and nuclear and cytoplasmic extraction kits were obtained from ThermoFisher Scientific (Waltham, MA). TGF β was purchased from R&D Systems (Minneapolis, MN). C-DIM8 and its analogs were synthesized in the laboratory. Primary antibodies (GAPDH, β -catenin, Non-phospho- β -catenin, p-SMAD2, SMAD2, p-SMAD3, SMAD3, Slug, ZO-1, ZEB1, Vimentin, and Bcl-2), Anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor® 488 Conjugate), HRP-linked secondary antibodies, DAPI, Bafilomycin A1, and Chloroquine were purchased from Cell Signaling Technology (Danvers, MA). NR4A1, Immunofluorescent-NR4A1, IL-24, and c-Myc antibodies were purchased from Abcam (Cambridge, UK). P84 antibody was purchased from GeneTex Inc (Irvine, CA). Formaldehyde, MG-132, β -actin antibody, M2 Flag antibody, β -catenin primers for PCR, and oligonucleotides for RNAi experiments (si β -catenin, siNR4A1, siIL-24, siBcl-2) were purchased from Sigma-Aldrich (St. Louis, MO). SMAD7 antibody was purchased from Novus Biologicals (Centennial, CO). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA) and human IL-24 cDNA clone in a pCMV-6 plasmid was purchased from Origene (Rockville, MD).

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siRNA interference/overexpression assay

RD or SMS-CTR cells (2.0×10^5) were seeded in medium supplemented with 10% FBS and were allowed to attach for 24 hours. The desired plasmids (siNR4A1, si β -catenin, siIL-24, siBcl-2 or pCMV-6) were then delivered into the cells at the concentration of 100 nM using Lipofectamine 2000 (50 μ mol/L). The excess Lipofectamine was removed after 6 hours of treatment by replacing the media. After 48-72 hours, cells were either lysed or treated with different reagents (C-DIM8, TGF- β) prior to lysis. The oligonucleotides used were as follows: siNR4A1_A, SASI_Hs02_00333289; siNR4A1_B SASI_Hs02_00333290; si β -catenin1, SASI_Hs02_00318698; si β -catenin2, SASI_Hs02_00318699; siIL-24_A, SASI_Hs01_00097938; siIL-24_B, SASI_Hs01_00097940; siBcl2 (1), SASI_Hs01_00119086; siBcl2 (2), SASI_Hs01_00119087.

Boyden chamber invasion assay

Attached cells (2.0×10^5) were treated with DMSO or with C-DIM8 prepared in medium supplemented with 2.5% charcoal-stripped FBS, for 24 hours, or were transfected with desired siRNAs/plasmids as outlined in siRNA interference assay. Cells were then co-treated with or without TGF- β (5 ng/ml) for 5 hours, trypsinized, and counted. One hundred thousand cells from each treatment condition were allowed to invade through the Boyden Chamber for 24 hours. Cells that invaded into the Boyden Chamber were fixed using formaldehyde, stained, and then counted. At least 3 replicates were performed for each treatment group.

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Immunofluorescence

Cells (2.0×10^5) were seeded and allowed to attach for 24 hours. They were then treated with DMSO or C-DIM8 for 24 hours (with or without 5 hours co-treatment with TGF- β), washed with PBS, fixed with 37% formaldehyde, permeabilized with 0.03% Triton, blocked with 5% BSA, and then treated with fluorescent-NR4A1 antibody for 24 hours. They were then washed with PBS and then treated with Anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor[®]488 Conjugate) antibody for an hour. They were then washed again with PBS and treated with 300 nM DAPI. After washing to remove excess DAPI, the location of NR4A1 (green) and nucleus (blue) in those cells were visualized using a fluorescent microscope (EVOS Cell Imaging Systems).

Nuclear/cytosolic extraction

Cells (2.0×10^5) were seeded in media supplemented with 10% FBS and were allowed to attach for 24 hours. They were then treated with DMSO or C-DIM8 prepared in 2.5% charcoal-stripped media (with or without 5 hours co-treatment with TGF- β). The nuclear and cytoplasmic extraction kit was then used according to the manufacturer's protocol to obtain cytosolic and nuclear fractions, which were further analyzed by western blots.

Western blot analysis

Cells (2.0×10^5) were seeded and after various treatments, whole cell lysates were obtained by treating them with high salt lysis buffer that contained protease and phosphatase inhibitors. The total protein in the lysates was quantified by Bradford assay. Equal amounts of protein from each lysate were then loaded on SDS polyacrylamide gel. The proteins on the gel were transferred to a PVDF membrane, which was blocked for an hour using 5% skimmed milk. The membrane was then incubated with primary antibody for 24 hours. It was then washed with Tris-buffered saline and Polysorbate 20 (TBST) and incubated with HRP-linked secondary antibody for 2 hours. The membrane was further washed with TBST and treated with Immobilon western chemiluminescence HRP-substrates to detect the protein bands using Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR, USA).

Polymerase chain reaction (PCR)

Cells (2.0×10^5) were seeded in a medium containing 10% FBS and were allowed to attach. After 24 hours, the medium was replaced with a fresh medium supplemented with 2.5% charcoal stripped FBS that also contained the desired compounds (DMSO, CDIM8, MG132 or their combination). The RNA was extracted from them after 24 hours using Zymo Research Quick-RNA Miniprep kit (Irvine, CA) by following the manufacturer's protocol. The Bio-Rad (Richmond, CA) iTaq Universal SYBER Green 1-step kit was then used and the manufacturer's instruction was followed to quantify the total β -catenin mRNA relative to TATA binding protein (TBP) mRNA which was used as a control. The β -catenin primer sequence used was: F: 5'-TCTGAGGACAAGCCACAAGATTACA-3' (sense) R: 5-TGGGCACCAATATCAAGTCCAA-3' (anti-sense).

Transmission electron microscopy

Cells (2.0×10^5) were seeded and were allowed to attach overnight on 35 mm Permanox petri dishes. They were then treated with either DMSO, or with C-DIM8 for 24 hours, and were rinsed with serum free media and then with 0.1 M cacodylate buffer, pH 7.2. They were then fixed with 2.5% paraformaldehyde, 2% glutaraldehyde, and 0.1 M cacodylate buffer, and stained with 1% osmium tetroxide, 0.2% ferrocyanide, and 0.1 M cacodylate buffer, followed by an hour of incubation with saturated uranyl acetate. The cells were then dehydrated using ethanol in an ascending series of concentration, and then infiltrated by incubation in 100% propylene oxide, followed by 1:1 propylene oxide:resin. They were then incubated in 100% resin for 16 hours, and were baked at 65°C for 48 hours. After that, blocks of the samples were cut and mounted to resin bullets, which were then cut into a 100 nm ultrathin sections using a Leica EM U6 ultramicrotome and were then placed on a copper grid (200 lines/inch hexagonal mesh). The sections were then post-stained with uranyl acetate for 6 minutes and then with Reynolds lead citrate for 45 seconds. These were then visualized using an FEI Morgagni 268 transmission electron microscope (operated by the Morgagni User Interface software), with a Megaview III CCD camera. The images were collected using iTEM software.

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ROS Measurements

Cells (2.0×10^5) were seeded in a medium supplemented with 10% FBS and were allowed to attach for 24 hours. They were then treated with DMSO or CDIM8 prepared in 2.5% charcoal stripped medium for 24 hours or transfected with the desired siRNAs, followed by the compound treatment for 24 hours. The levels of ROS were then measured with Accuri flow cytometer using the cell permeable H2D-CFDA reagent as outlined in the manufacturer's instruction (Life Technologies Inc.).

Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences between the treatment groups. Each experiment was repeated three times and the results were presented as means with error bars representing 95% confidence intervals. Data with a *P* value of <0.05 were considered statistically significant.

Results

TGF β induces invasion of ERMS cells which is inhibited by NR4A1 antagonists

TGF β enhances invasion of late stage tumors [25, 26], induces growth and inhibits differentiation of ERMS cells [21-24]. Previous studies in breast and lung cancer cells show that this response was NR4A1-dependent and could be inhibited by bis-indole derived NR4A1 antagonists [17, 18]. Results illustrated in **Figure 1A** show that TGF β induces invasion of RD and SMS-CTR ERMS cells whereas it did not affect invasion of Rh30 ARMS cells (data not shown). The effects of the NR4A1 antagonist CDIM8 on TGF β -induced invasion was determined and CDIM8 alone inhibited invasion as well as TGF β -mediated invasion of RD and SMS-CTR cells (**Figure 1A**). These results demonstrate the effects of TGF β on ERMS (but not ARMS) cell invasion and therefore TGF β -induced responses in ERMS cells include induction of cell growth and invasion, and inhibition of differentiation.

NR4A1 is extranuclear in RD and SMS-CTR cells and does not affect TGF β -induced SMAD signaling

TGF β -induced invasion of lung and breast cancer cells resulted in nuclear export of NR4A1

and subsequent degradation of SMAD7 [17, 18], however, results in **Figure 1B** demonstrate that NR4A1 protein is primarily cytosolic in RD and SMS-CTR cells and TGF β does not affect NR4A1 protein levels whereas these are decreased after treatment with CDIM8. Moreover, treatment with TGF β , CDIM8 or their combination did not affect the intracellular location of NR4A1 which remained extranuclear. We further confirmed the location of NR4A1 in RD (**Figure 1C**) and SMS-CTR (**Figure 1D**) cells by immunostaining and showed that in untreated or treated cells, NR4A1 remained extranuclear and exhibited perinuclear staining. TGF β induced nuclear export of NR4A1 in breast and lung cancer cells [17, 18] and this receptor formed part of a proteasome complex that degraded inhibitory SMAD7. This response was inhibited by CDIM8, as it blocked the nuclear export of NR4A1. SMAD7 plays an inhibitory role in TGF β -induced responses by enhancing degradation of the TGF β receptor [27]. In contrast, the treatment of RD cells with CDIM8 alone, CDIM8 plus TGF β , MG-132 (proteasome inhibitor) alone, and MG-132 plus TGF β had minimal effects on expression of SMAD7 as the protein levels of SMAD7 were either unchanged or decreased after the treatment (**Figure 2A**). Similar results were observed in SMS-CTR cells (**Figure 2B**) demonstrating that the role of NR4A1 and effects of the NR4A1 antagonists on TGF β -induced invasion was largely independent of their effects on expression of inhibitory SMAD7. These results indicate that in ERMS cells, CDIM8 did not enhance nuclear retention of NR4A1 or decrease SMAD7 degradation suggesting that the TGF β -NR4A1-SMAD7 pathway observed in breast and lung cancer cells is inoperative in ERMS cells. TGF β -dependent activation of SMAD2/SMAD3 can also play a role in enhanced invasion however, although TGF β activated (phosphorylated) SMAD2 and SMAD3 in RD (**Figure 2C**) and SMS-CTR (**Figure 2D**) cells, CDIM8 alone affected neither the SMAD phosphorylation, nor the TGF β -induced responses, indicating that these effects are NR4A1-independent.

β -catenin is downregulated by CDIM8

NR4A1 interacts with β -catenin [28, 29] and in breast cancer cells, CDIM8 blocks TGF β -induced nuclear localization of β -catenin resulting in accumulation of β -catenin in the cytosol, which then undergoes proteasome-dependent

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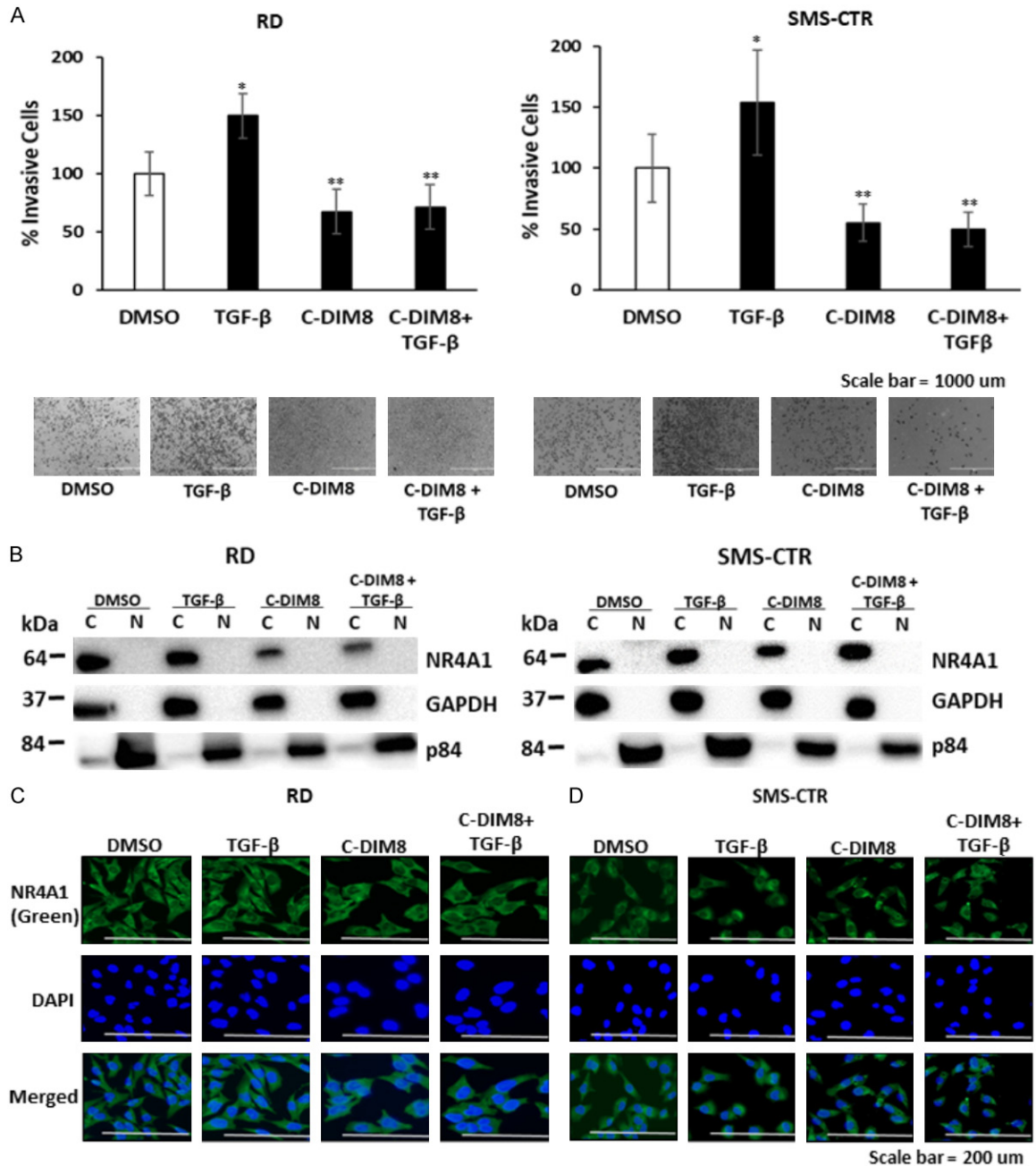


Figure 1. Modulation of TGF β -induced invasion of RMS cells and subcellular localization of NR4A1 in ERMS cells. (A) RD and SMS-CTR cells were treated with 5 ng/ml TGF β , 20 μ M CDIM8, or their combination and cell invasion was determined in a Boyden chamber assay as outlined in the Methods. (B) RD and SMS-CTR cells were treated with DMSO, TGF β , CDIM8 and their combination and cytosolic (C) and nuclear (N) fractions were separated and analyzed by western blots as outlined in the Methods. RD (C) and SMS-CTR (D) cells were treated as described above (A, B) and cellular location of NR4A1 was determined by DAPI (for nuclear staining) and NR4A1 antibody staining by immunofluorescence analysis as outlined in the Methods.

degradation [19]. Since β -catenin is also involved in epithelial-mesenchymal transition (EMT) and cell invasion, we transfected RD and SMS-CTR cells with siNR4A1 to silence NR4A1 (Figure 3A) and also treated these cells with

CDIM8 (Figure 3B) and showed that levels of total β -catenin were decreased by both NR4A1 knockdown and the NR4A1 antagonist (Relative band intensity quantification is illustrated in Figure S1A and S1B). Similar effects on

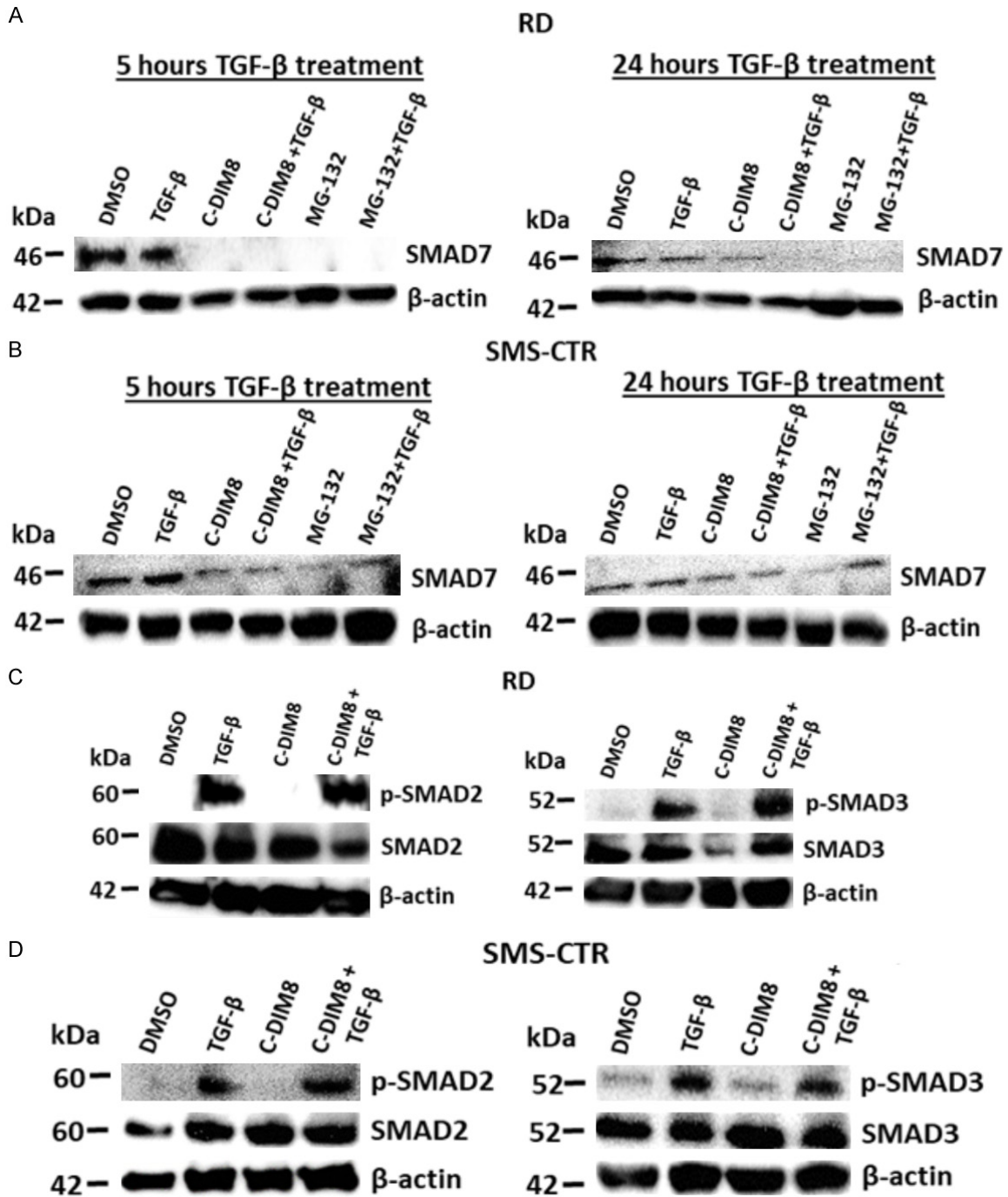


Figure 2. Effects of TGF β /CDIM8 on SMADS. RD (A) or SMS-CTR cells (B) were treated with DMSO or 5 μ M MG132 for 3 hours alone or in combination with 20 μ M CDIM8 or with 5 ng/ml TGF β for either 5 or 24 hours and whole cell lysates were analyzed by western blots as outlined in the Methods. RD (C) and SMS-CTR (D) cells were treated with DMSO, 5 ng/ml TGF β , 20 μ M CDIM8 alone or in combination for 24 hours and whole cell lysates were analyzed by western blots as outlined in the Methods.

β -catenin were previously observed in breast cancer cells when NR4A1 was silenced or inactivated by CDIM8 [17]. Western blot analysis of the subcellular location of β -catenin showed

that in untreated RD (Figure 3C) and SMS-CTR cells (Figure 3D), β -catenin was located in both the cytosolic and nuclear fractions of both cell lines with higher cytosolic and nuclear β -catenin

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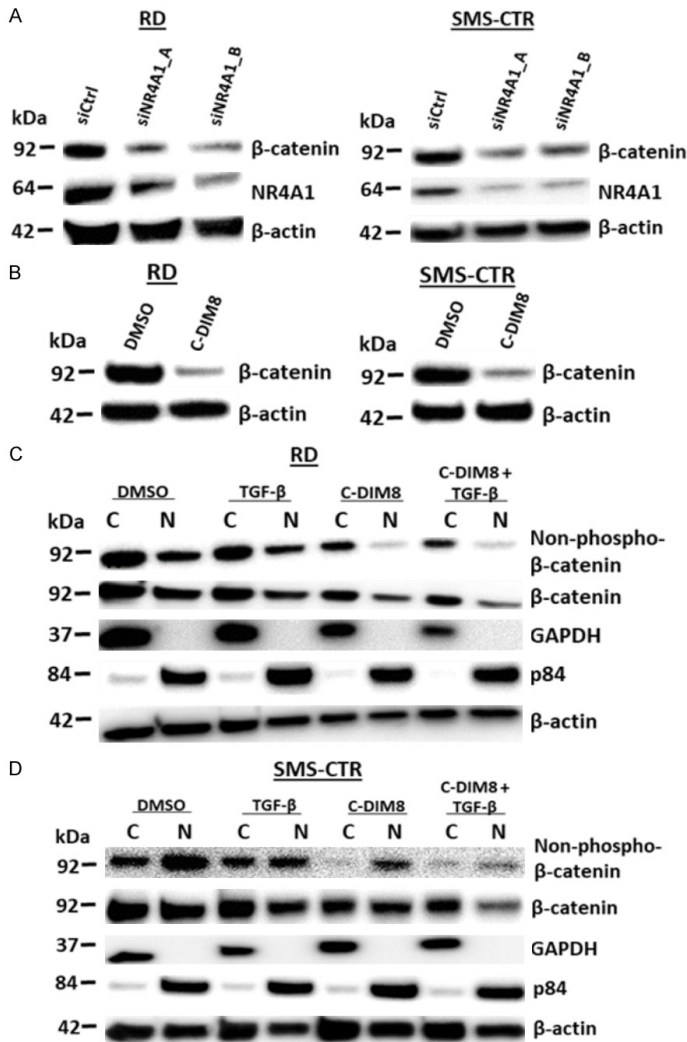


Figure 3. Effects of NR4A1 and TGF β on β -catenin expression. RD and SMS-CTR cells were treated with siNR4A1 (A) or treated with CDIM8 for 24 hours (B) and whole cell lysates were analyzed by western blots as outlined in the Methods. RD (C) and SMS-CTR (D) cells were treated with 5 ng/ml TGF β and various drug combinations and cytosolic and nuclear extracts were obtained and analyzed by western blots as outlined in the Methods.

levels in RD and SMS-CTR cells respectively. TGF β alone did not decrease or change the sub-cellular location of β -catenin or its active non-phosphorylated form. CDIM8 alone or in combination with TGF β decreased expression of cytosolic and nuclear β -catenin (total and non-phosphorylated) in both cell lines however, in SMS-CTR cells treated with CDIM8, the non-phosphorylated form was primarily nuclear indicating some differential loss of cytosolic β -catenin.

Further examination of the effects of CDIM8 on β -catenin and downstream targets was investi-

gated and in RD and SMS-CTR cells (Figure 4A and 4B), CDIM8 alone or in combination with TGF β decreased expression of β -catenin (total and non-phosphorylated) and its downstream targets (c-Myc and Slug) and the EMT marker ZEB1. We also observed that TGF β alone had minimal effects on c-Myc, Slug, ZO-1 and ZEB1 in ERMS cells (Figure 4A and 4B) and this may be due to the high endogenous levels of these gene products. These results demonstrate that NR4A1 silencing or ligand-induced inactivation of NR4A1 downregulated β -catenin (Relative band intensities are quantified and illustrated in Figure S1C and S1D). However, in contrast to a similar response in breast cancer cells [17], decreased expression of β -catenin due to CDIM8 was proteasome-independent and was not affected by co-treatment with the proteasome inhibitor MG132 (Figures 4C and S2A). Furthermore, this response was also lysosome-independent and remained unaffected by co-treatment with the lysosome inhibitors Bafilomycin A1 and Chloroquine (Figure S2B). Since β -catenin plays a role in EMT and cell invasion, we investigated the effects of β -catenin silencing (Figure 4D) on basal and TGF β -induced invasion of RD and SMS-CTR cells (Figure 4E). The results show that β -catenin silencing results in decreased basal and TGF β -induced invasion of RD and SMS-CTR cells suggesting that CDIM8-induced downregulation of β -catenin expression contributes to

the inhibitory effects of CDIM8 on RMS cell invasion.

Role of IL-24 and the NR4A1-Bcl-2 complex

A recent study reported that CDIM8 induced the tumor suppressor like cytokine IL-24 in RMS (RD and Rh30) cells [30] and IL-24 exhibits a diverse spectrum of anticancer activities including inhibition of cancer cell invasion [30-35]. Since knockdown of β -catenin inhibits ERMS cell invasion (Figure 4E), we investigated whether induction of IL-24 by CDIM8 was associated with RMS cell invasion and if this was

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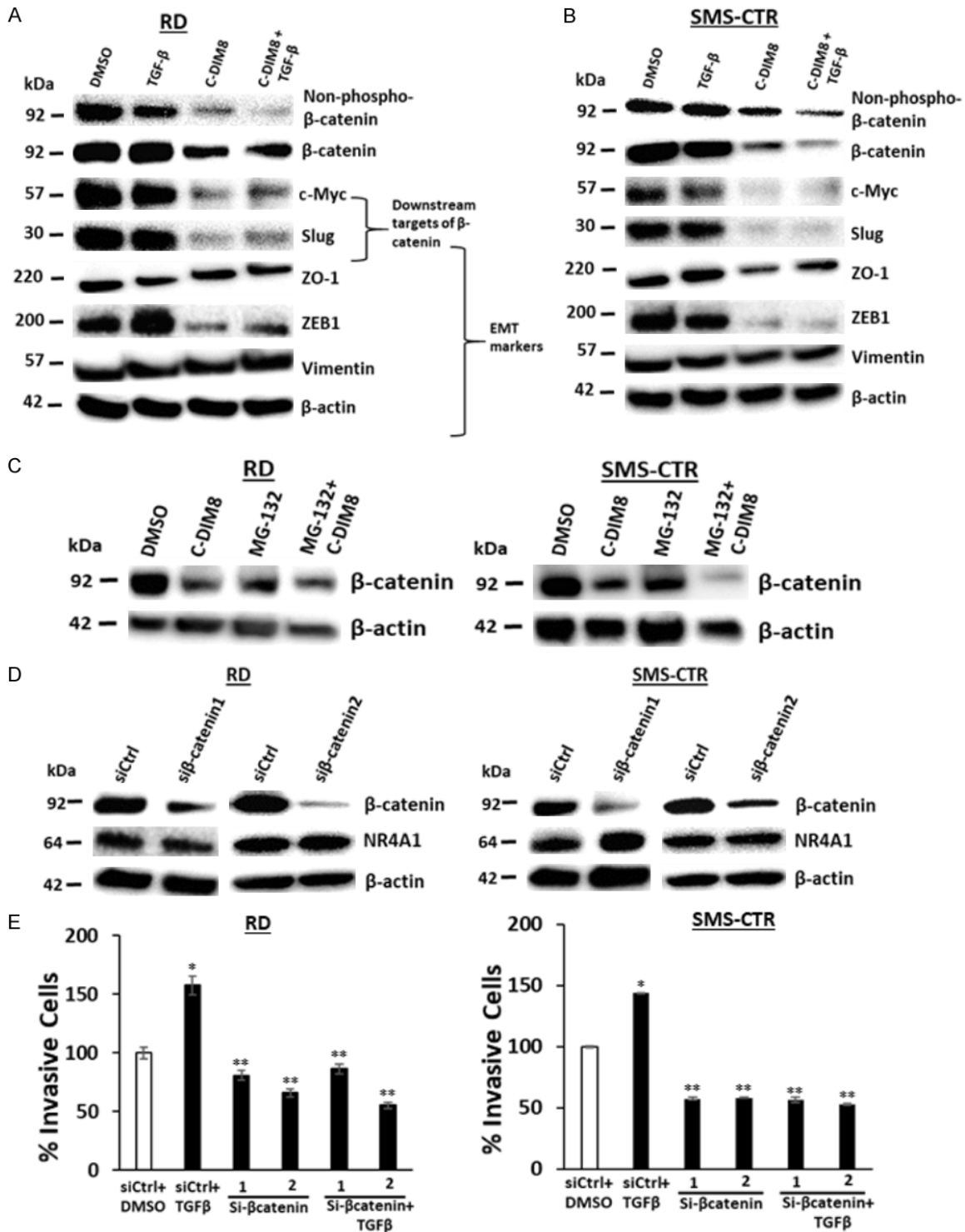


Figure 4. Regulation of β -catenin expression by TGF β and NR4A1 antagonists. RD (A) and SMS-CTR (B) cells were treated with DMSO, TGF β , CDIM8 or their combination for 24 hours and whole cell lysates were analyzed by western blots as outlined in the Methods. (C) RD and SMS-CTR were treated with DMSO, 20 μ M CDIM8, 5 μ M MG132 (3 hours) or their combination for 24 hours and whole cell lysates were analyzed by western blots as outlined in the Methods. RD and SMS-CTR cells were transfected with si β -catenin alone (D) or in combination with TGF β (E) and whole cell lysates were analyzed by western blots (D) or effects on cell invasion (E) were determined in a Boyden chamber assay respectively as outlined in the Methods. Results (E) are means \pm SD for at least 3 separate determinations as significant (P<0.05) induction (*) or inhibition (**) of the induced response are indicated.

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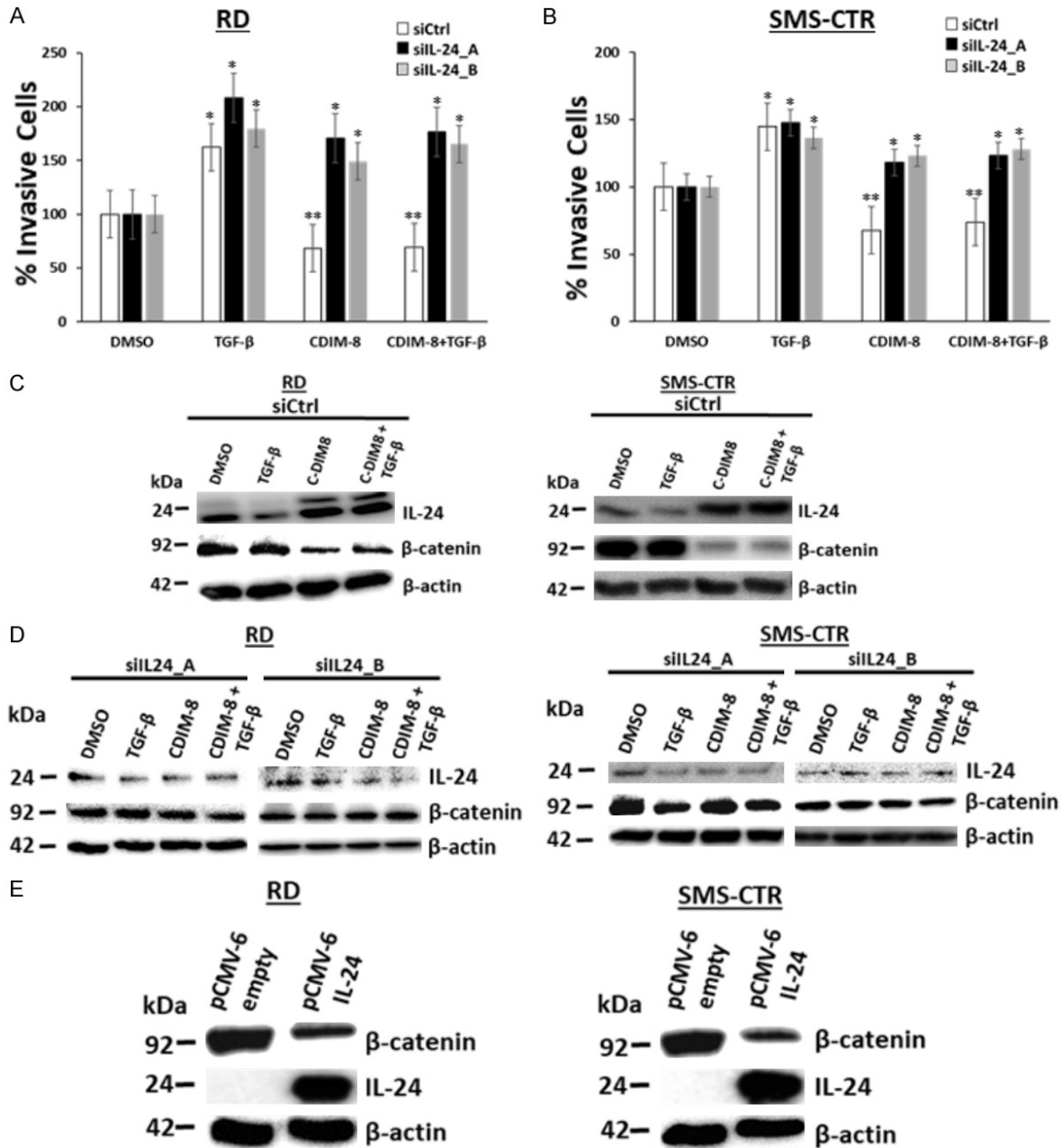


Figure 5. Role of IL-24 in the inhibition of TGFβ-induced invasion by NR4A1 antagonists. RD (A) and SMS-CTR (B) cells were transfected with siCtrl (non-specific oligonucleotide) or siIL-24 and treated with TGFβ, CDIM8 or their combination and effects on cell invasion were determined in a Boyden chamber assay as outlined in the Methods. RD and SMS-CTR cells were transfected with a control oligonucleotide (siCtrl) (C) or siIL-24 (D) and whole cell lysates were analyzed by western blots. (E) RD and SMS-CTR cells were transfected with an IL-24 expression plasmid and after 48 hours whole cell lysates were analyzed by western blots. Results (A/B) are means ± SD of at least 3 separated determinations and significant ($P < 0.05$) induction (*) or inhibition (**) of invasion is indicated.

due, in part, to down regulation of β-catenin. Results in **Figure 5A** and **5B** show that in RD and SMS-CTR cells treated with TGFβ, CDIM8 alone, or CDIM8 plus TGFβ, knockdown of IL-24 (siIL-24) enhanced invasion in all 3 treatment groups suggesting a role for IL-24 as an inhibitor of invasion. Western blot analysis of the

various treatment groups shows that CDIM8 and CDIM8+TGFβ-induced IL-24 and decreased β-catenin expression (**Figure 5C**) and this correlated with decreased basal and TGFβ-induced invasion (**Figure 5A** and **5B**). In contrast, western blot analysis of cell lysates from these same treatments (CDIM8 and CDIM8+TGFβ) in

combination with IL-24 silencing showed that β -catenin levels were not decreased in the absence of IL-24 (**Figure 5D**) and this correlated with enhanced invasion after IL-24 silencing (**Figure 5A** and **5B**). Relative band intensities for **Figure 5C** and **5D** are quantified in [Figure S3](#). These results suggest that induction of IL-24 by CDIM8 was important for inhibiting ERMS cell invasion and for downregulation of β -catenin, and this complements the data showing that β -catenin silencing inhibits TGF β -induced invasion of ERMS cells (**Figure 4E**). Previous studies in pancreatic and breast cancer cells show that overexpression of IL-24 decreased expression of β -catenin protein [36, 37] and using a similar approach, we also demonstrate that overexpression of IL-24 in ERMS cells decreased β -catenin expression (**Figure 5E**).

Thus, our results indicate that inhibition of basal and TGF β -induced invasion of RD and SMS-CTR cells is due to induction of IL-24 and its subsequent downregulation of β -catenin. IL-24 is induced by ROS in cancer cell lines [38] and several studies show that treatment of some cancer cell lines with apoptosis-inducing agents results in nuclear export of NR4A1 and interaction with Bcl-2 to form a complex that disrupts mitochondria and induces ROS [39-45]. Thus, we hypothesized that induction of IL-24 by CDIM8 in RD cells may be due to activation of the extranuclear Bcl-2-NR4A1 complex and this was further investigated using RD cells as a model. **Figure 6A** shows that oligonucleotides that target Bcl-2 decrease expression of the target gene product in RD cells. Treatment of RD cells with CDIM8 induces IL-24 and this response is blocked after co-treatment with GSH thus confirming a role for ROS in the induction of IL-24 in RD cells (**Figure 6B**). The role of Bcl-2 in this process is supported by results in **Figure 6C** showing that induction of IL-24 by CDIM8 in RD cells is decreased after knockdown of Bcl-2. Using the same treatment protocol, we also observed that CDIM8 induces ROS compared to treatment with solvent (DMSO) or control oligonucleotide, however, in Bcl-2-silenced RD cells induction of ROS by CDIM8 is abrogated (**Figure 6D**). The results suggest that CDIM8-mediated induction of IL-24 and ROS are Bcl-2 dependent and therefore we examined the effects of CDIM8 on mitochondria by electron microscopy. Control RD cells exhibit the typical mitochondrial structure where-

as after treatment with CDIM8 we observed extensive evidence of misshapen mitochondria and mitochondrial damage (**Figure 6E**) and this was similar to previous studies using apoptosis-inducing agents. Thus, in RD cells where NR4A1 is extranuclear, CDIM8 activates the Bcl-2-NR4A1 complex to induce mitochondrial damage, ROS and IL-24 which downregulates β -catenin thereby inhibiting basal and TGF β -induced invasion of RMS cells.

Discussion

TGF β plays a paradoxical role in cancer where many early stage tumors are inhibited by this growth factor whereas it enhances migration and invasion of late stage cancers [28-30]. The mechanisms associated with TGF β -induced cancer cell invasion are complex and can involve multiple pathways including formation of activated SMAD2/SMAD3-SMAD4 complexes. Another mechanism linked to TGF β -induced invasion is associated with degradation of the inhibitory SMAD7 which inhibits TGF β signaling by induction of TGF β receptor 1 degradation [27]. Recent studies have shown that NR4A1 plays an important role in TGF β -regulated SMAD7 degradation in breast and lung cancer cells [3, 17, 18]. TGF β induces phosphorylation of NR4A1 which is necessary for nuclear export of NR4A1 and this receptor forms a complex with Arkadia, RNF12, Axin2 and SMAD7 resulting in proteasome-dependent degradation of SMAD7 [3, 17, 18]. In this study, we initially showed that TGF β also induces invasion of RD and SMS-CTR (ERMS) but not Rh30 (ARMS) cells and this is consistent with previous studies showing that TGF β primarily enhances carcinogenesis of ERMS cells [21-28]. Moreover, we also show that the NR4A1 antagonist CDIM8 inhibits basal and TGF β -induced invasion of ERMS cells (**Figure 1**). However, although CDIM8 inhibits TGF β -induced invasion in breast and lung cancer cells and also in ERMS cells (**Figure 1**), the mechanism of the inhibitory effects in ERMS cells is distinct from that previously observed in lung and breast cancer cells [3, 17, 18]. A major difference involves the intracellular location of NR4A1 in ERMS cells; the receptor is cytosolic/perinuclear and treatment with CDIM8, TGF β alone or in combination with CDIM8 does not induce changes in the intracellular location of NR4A1 (**Figure 1C** and **1D**). Moreover, CDIM8 does not block TGF β -induced degradation of SMAD7 or affect

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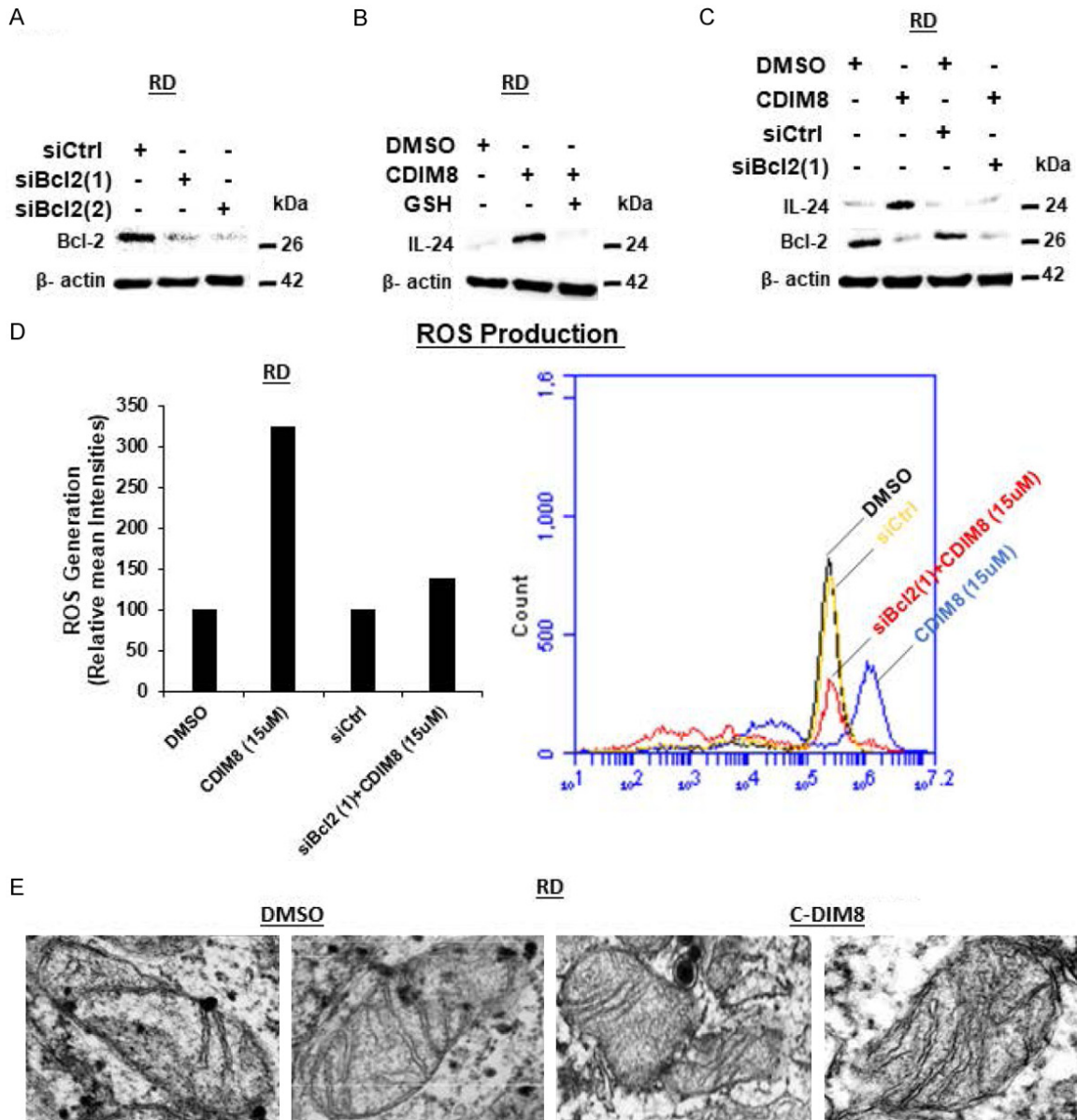


Figure 6. NR4A1-Bcl-2 interactions and mitochondrial damage. RD cells were transfected with siCtrl/siBcl-2 oligonucleotides (A), treated with DMSO, CDIM8 alone or in combination with GSH (5 mM) (B) or transfected with siCtrl/siBcl-2 and treated with DMSO or CDIM8 (C) and at the end of the treatment period whole cell lysates were analyzed by western blots. (D) RD cells were treated with DMSO, CDIM8 (15 μM), siCtrl or siBcl-2/CDIM8 and the levels of ROS were determined as outlined in the Materials and Methods. (E) Electron microscopic analysis of mitochondria in RD cells treated with DMSO or 15 μM CDIM8 was determined as outlined in the Methods.

activation of SMAD2 or SMAD3. This indicates that other pathways are involved in TGFβ-mediated ERMS cell invasion.

Previous reports have demonstrated interactions between NR4A1 and β-catenin [28, 29] and β-catenin is involved in epithelial to mesenchymal transition (EMT) and cell invasion [46-48]. Our results indicate that β-catenin is highly

expressed and distributed in the cytosol and nucleus of ERMS cells and although TGFβ has minimal effects on β-catenin levels, the NR4A1 antagonist CDIM8 alone or in combination with TGFβ decreases expression of β-catenin and downstream genes (Figures 3 and 4). A previous study showed that NR4A1 activated proteasome-dependent degradation of β-catenin [28]; however, we did not observe any protec-

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tion from CDIM8-induced downregulation of β -catenin by the proteasome inhibitor MG132 (**Figure 4C**). It was also reported that NR4A1 interacted with β -catenin in colon cancer cells and this resulted in proteasome-dependent degradation of β -catenin [28] whereas in ERMS cells, both NR4A1 antagonists and NR4A1 silencing decreased β -catenin expression (**Figure 4A** and **4B**) and this was proteasome independent (**Figure 4C**).

A recent study in this laboratory [30] reported that NR4A1 silencing or antagonism by CDIM8 induced the tumor suppressor-like cytokine IL-24 in RMS cells and it has previously been reported that IL-24 inhibits cancer cell migration, invasion and metastasis [31-35]. In ERMS cells, inhibition of TGF β -induced invasion by CDIM8 is accompanied by induction of IL-24, and decreased expression of β -catenin; moreover, the effects of the NR4A1 antagonist on invasion, IL-24 expression and β -catenin degradation were attenuated by IL-24 silencing (**Figure 5**). These results, coupled with the effects of IL-24 on β -catenin expression (decreased) (**Figure 5E**) suggest that the inhibition of basal and TGF β -induced ERMS cell invasion by CDIM8 is due, in part, to the induction of IL-24 and IL-24-mediated downregulation of β -catenin (**Figure 5E**). In addition, we show that CDIM8 activates the NR4A1-Bcl-2 complex to disrupt mitochondria and induce ROS and IL-24 (**Figure 6**) and this complements previous studies showing the effects of this complex on mitochondria [37-43]. This represents a novel pathway for inhibiting late stage TGF β -induced cancer cell invasion and current studies are focused on the mechanism of β -catenin downregulation by IL-24 and potential clinical applications of drug-induced IL-24 for inhibiting invasion of ERMS and other cancer cell lines and their associated tumors *in vivo*.

In summary, this study demonstrates for the first time that TGF β induces invasion of ERMS cells and this response is inhibited by the NR4A1 antagonist CDIM8. In contrast to other solid tumors, NR4A1 in ERMS cells is extranuclear and TGF β -NR4A1 crosstalk is independent of nuclear export of the receptor or modulation of SMAD signaling. The cross talk between NR4A1 and other growth factors/cytokines and effects of CDIM8 in ERMS cells has not been determined and is currently being investigated. In ERMS cells, NR4A1 regulates

expression of β -catenin and therefore NR4A1 silencing or treatment with an NR4A1 antagonist decreases β -catenin expression and β -catenin-linked EMT and invasion. We also observed that β -catenin downregulation by NR4A1 inactivation involves induction of the tumor suppressor-like cytokine IL-24. Thus, our results demonstrate that basal and TGF β -induced invasion of ERMS cells are targetable by NR4A1 antagonists which inactivate the pro-invasion TGF β -NR4A1- β -catenin pathway by induction of IL-24. Thus, the bis-indole derived NR4A1 antagonist inhibits TGF β -induced and basal ERMS cell invasion through a novel pathway involving extranuclear NR4A1/Bcl-2 which induces ROS and IL-24. IL-24 decreases β -catenin expression and β -catenin-dependent invasion. Previous studies show that CDIM8 and related NR4A1 antagonists bind nuclear NR4A1 and either inactivate NR4A1-regulated pro-oncogenic genes pathways [5, 11-18] or inhibit the effects of TGF β by blocking nuclear export of NR4A1 [3, 17, 18]. In contrast, this study shows that in ERMS cells, where NR4A1 is extranuclear, CDIM8 activates the pro-apoptotic NR4A1-Bcl2 pathway which also induces IL-24 indicating the anticancer activities of CDIM8 are linked to targeting of either the nuclear or the extranuclear NR4A1 receptor.

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Disclosure of conflict of interest

None.

Abbreviations

ARMS, alveolar rhabdomyosarcoma; CDIM8, 1,1-bis(3'-indolyl)-1-(p-hydroxyphenyl)methane; EMT, epithelial-mesenchymal transition; ERMS, embryonal rhabdomyosarcoma; FBS, fetal bovine serum; IL-24, interleukin-24; NR4A, nuclear receptor 4A; PBS, phosphate-buffered saline; RMS, rhabdomyosarcoma; ROS, reactive oxygen species; siRNA, small inhibitory RNA; TBST, tris buffered saline and polysorbate 20; TGF β , transforming growth factor beta.

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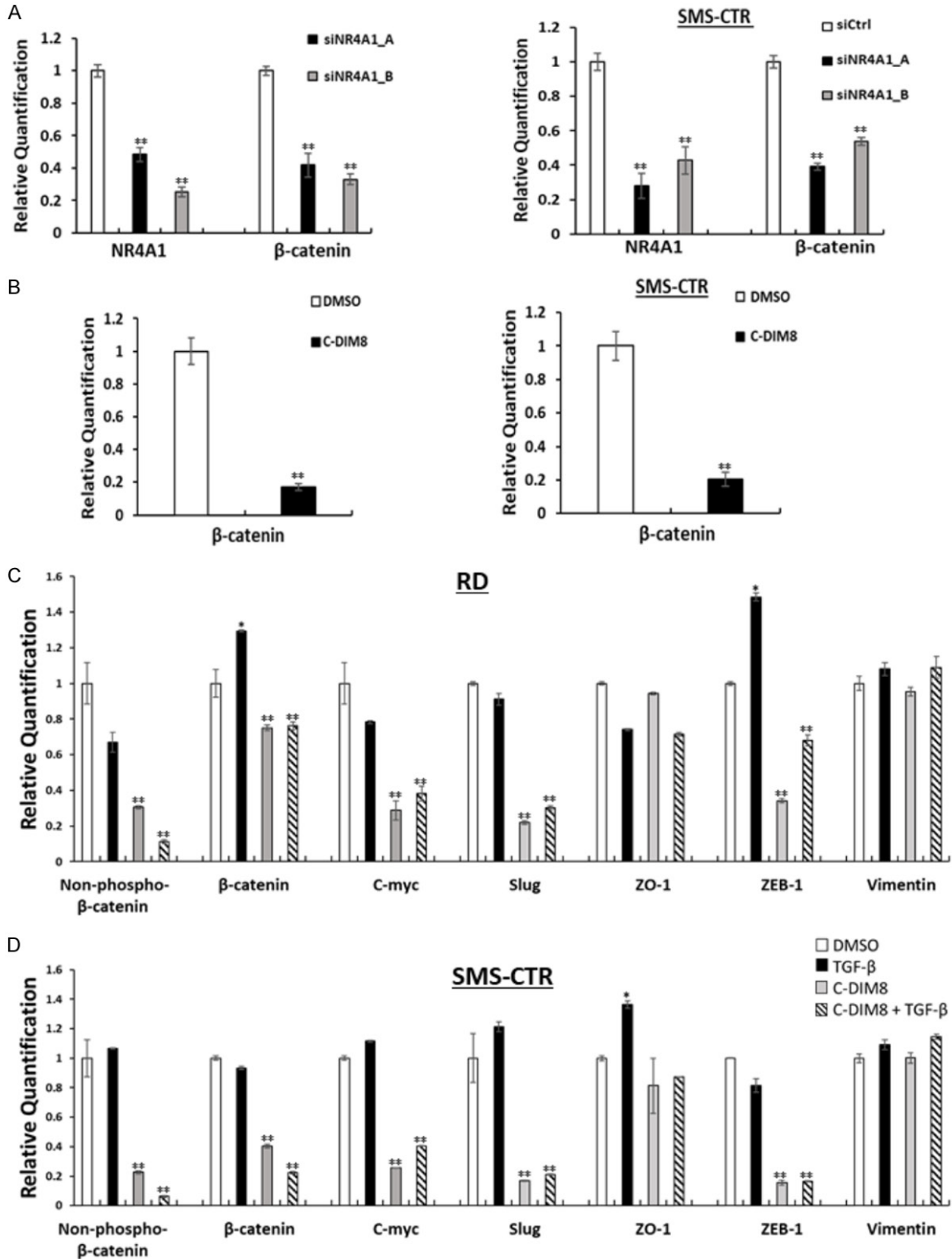


Figure S1. Quantification of protein levels in **Figures 3A, 3B, 4A** and **4B**. Levels of (A) NR4A1 and β-catenin (From **Figure 3A**), (B) β-catenin (From **Figure 3B**) and (C, D) β-catenin and EMT markers (From **Figure 4A** and **4B**) in RD and SMS-CTR cells after different treatment conditions as indicated were quantitated relative to control and normalized to β-actin. Results are means ± SD of at least three separate determinations and significant ($p < 0.05$) induction (*) or inhibition (**) of an induced response are indicated.

NR4A1 antagonists inhibit invasion of RMS cells

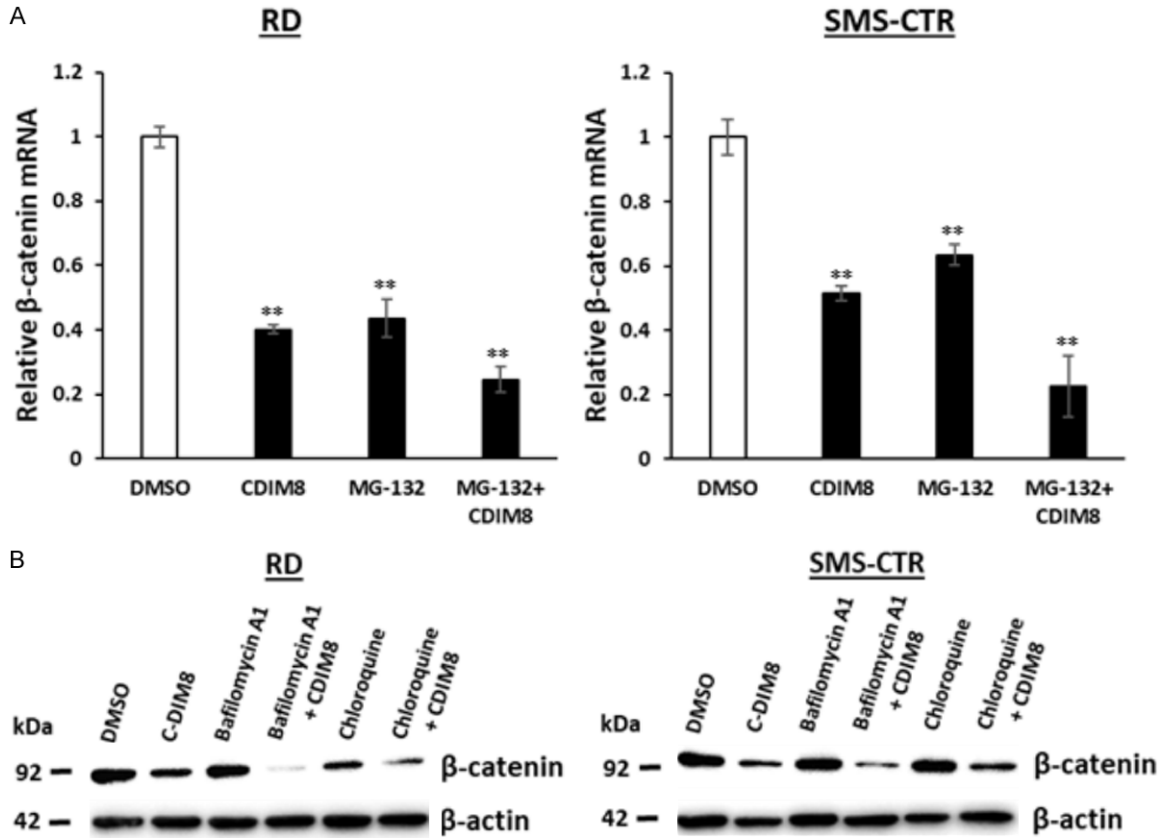


Figure S2. β -catenin downregulation by CDIM8 is proteasome and lysosome independent. A. RD and SMS-CTR were treated with DMSO, 20 μ M CDIM8, 5 μ M MG132 (3 hours) or their combination for 24 hours and the effect on β -catenin mRNA level was determined by real time PCR as outlined in the Methods. B. RD and SMS-CTR cells were treated with DMSO, 20 μ M CDIM8, 1 μ M Bafilomycin A1, 50 μ M Chloroquine, or their combination for 24 hours and the effect on β -catenin protein level was determined by western blot as outlined in Methods. Results are expressed as means \pm SD for at least 3 replicated determinations for each treatment group and significant ($P < 0.05$) decreased (**) response are indicated.

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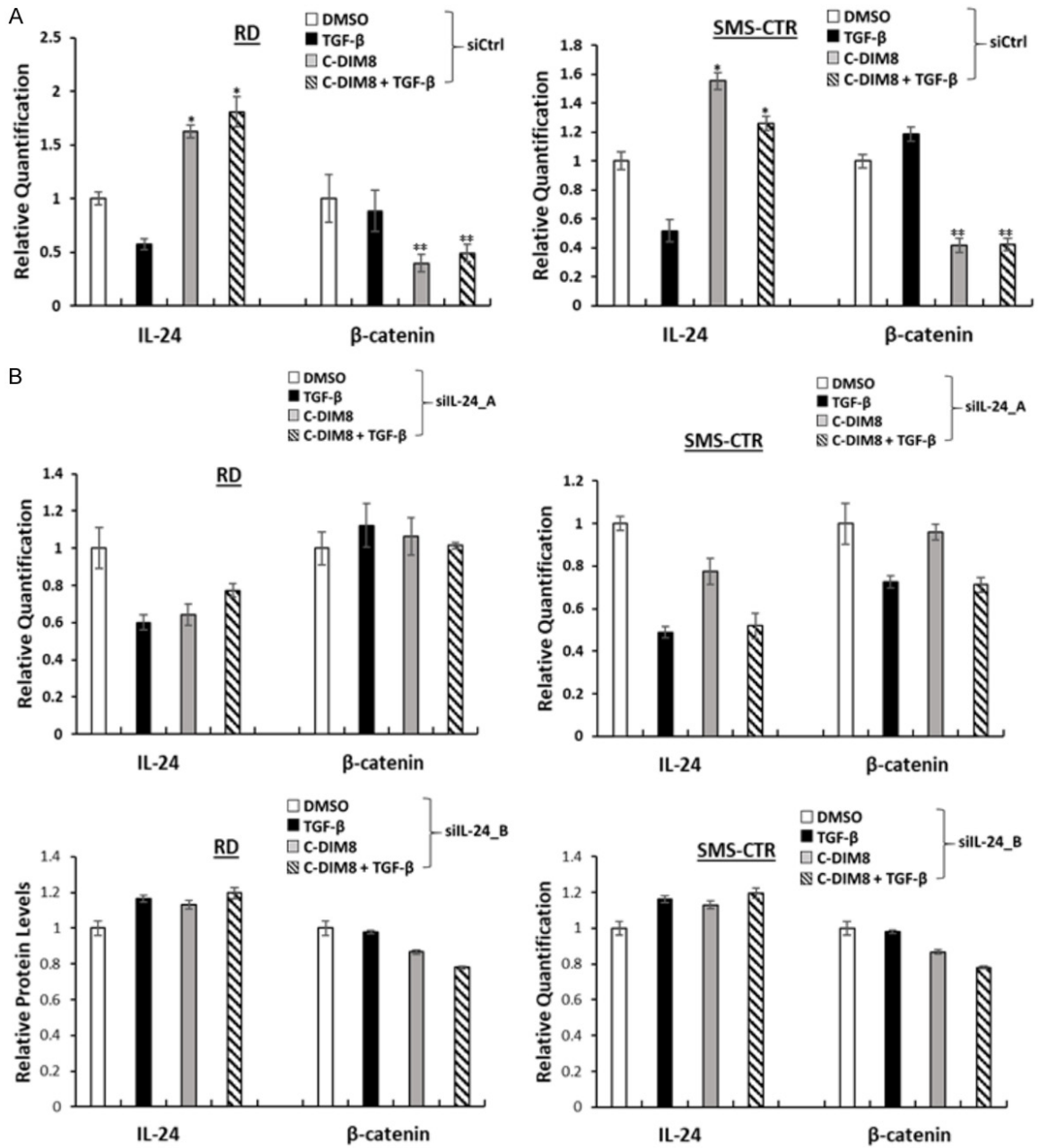


Figure S3. Quantification of protein levels in Figures 5C and 5D. Levels of IL-24 and β-catenin in RD and SMS-CTR cells transfected with (A) siCtrl (From Figure 5C) or (B) silL-24 (From Figure 5D) were quantitated relative to control and normalized to β-actin. Results are means ± SD of at least three separate determinations and significant (P<0.05) induction (*) or inhibition (**) of the induced response are indicated.